Virology 291, 68–76 (2001)
doi:10.1006/viro.2001.1219, available online at http://www.idealibrary.com on IDEAL®

Analysis of Early Region 1 of Porcine Adenovirus Type 3[1]

Yan Zhou[2] and Suresh K. Tikoo[3]

Virology Group, Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E3, Canada

Received June 21, 2001; returned to author for revision July 30, 2001; accepted October 1, 2001

To identify the proteins encoded by the porcine adenovirus 3 (PAV-3) E1 region, rabbit antisera were prepared using a bacterial fusion protein encoding E1A, E1B^{small}, or E1B^{large} protein. Sera against E1A, E1B^{small}, and E1B^{large} immunoprecipitated a protein of 35, 23, and 53 kDa, respectively, in *in vitro* translated and transcribed mRNA and PAV-3 infected cells. To determine the role of E1 proteins in PAV-3 replication, we constructed vectors with a deletion(s) in the E1 region. Mutant PAV211, containing deletions in E1A and E3, grew to titers similar to wild-type in VIDO R1 cells (E1A complementing) but not in swine testicular (ST) cells. No early protein (E1B^{small}, DNA binding protein) expression could be detected in PAV211 infected ST cells by Western blots. Mutant PAV212, containing deletions in E1B^{small} and E3, grew to wild-type titers in VIDO R1 or ST cells. These deletions were successfully rescued, resulting in recombinant PAV214, containing deletions in E1A, E1B^{small}, and E3. However, mutant PAV-3, containing a triple stop codon inserted in the E1B^{large} coding sequence, could not be isolated. Next, we constructed a recombinant PAV216 by inserting the green fluorescent protein gene flanked by a promoter and a poly(A) in the E1A region of the PAV214 genome. Both PAV214 and PAV216 replicate as efficiently as wild-type in VIDO R1 cells. These results suggested that (a) E1A is essential for virus replication and is required for the activation of other PAV-3 early genes, (b) E1B^{small} is not essential for replication of PAV-3, and (c) E1B^{large} is essential for virus replication. Moreover, the PAV216 vector can be used for the expression of a transgene.

Key Words: porcine adenovirus-3; E1A; E1B^{small}; E1B^{large}; PAV-3 vector.

INTRODUCTION

Adenoviruses have been studied for several decades to probe the process of oncogenesis as they are able to induce tumors in animals and transform cultured cells (Darbyshire, 1966; Graham, 1984; Graham *et al.,* 1977; Houweling *et al.,* 1980; Jones and Shenk, 1979). Since the first use of human adenovirus as a gene transfer vector *in vivo* (Ballay *et al.,* 1985), the utility of adenoviruses as vectors for gene therapy and vaccination has been the subject of intense exploration. Several features of adenoviruses have made them attractive candidates for use as gene delivery vectors (Hitt and Graham, 2000; Imler, 1995). Adenoviruses can be grown to high titers in cell culture and can transduce both dividing and nondividing cells without inserting themselves into the host cell's genome. However, vectors derived from adenoviruses that naturally infect and replicate in humans may not be the ideal candidates for human therapeutic purposes. Since some adenoviruses are endemic in most populations, there is a risk that the recombinant human adenovirus (HAV) vectors could recombine with a preexisting HAV and generate a replication-competent adenovirus (RCA) (Hehir *et al.,* 1996; Mastrangeli *et al.,* 1996). In addition, preexisting immunity against HAV can limit the initial transduction by HAV vectors or might enhance the cellular immune response against transduced cells. To overcome some of the problems associated with the use of human adenovirus as vector, we (Reddy *et al.,* 1999b, 1999c; Zakhartchouk *et al.,* 1998, 1999) and others (Khatri *et al.,* 1997; Klonjkowski *et al.,* 1997; Michou *et al.,* 1999; Sheppard *et al.,* 1998; Xu *et al.,* 1997) are exploring the utility of nonhuman adenoviruses as expression vectors for animal vaccination and human gene therapy.

Porcine adenoviruses (PAVs) belong to the *Mastadenovirus* genus of the *Adenoviridae* family. Of the five serotypes identified so far (Derbyshire *et al.,* 1975; Hirahara *et al.,* 1990), serotype 3 (PAV-3) could propagate to high titers in cell culture (Hirahara *et al.,* 1990). Recently, the transcription map and complete DNA sequence of the PAV-3 genome were reported (Reddy *et al.,* 1998a,b). Using the *Escherichia coli* BJ 5183 recombination system (Chartier *et al.,* 1996), we have successfully constructed a full-length infectious clone of the PAV-3 genome (Reddy *et al.,* 1999a) and recombinant PAV-3s (Reddy *et al.,* 1999a,b).

Since the E1A region has oncogenic potential (Darbyshire, 1966; Whyte *et al.,* 1988), recombinant adenoviruses are made replication-defective by the deletion of the E1A and E1B region. In addition to removing the risk of transformation (Graham, 1984), deletion of the E1A region reduces the expression of early and late region genes, resulting in a lower immune response to adeno-

[1] Published as VIDO Journal Series No. 290.

[2] Present address: Department of Oncology, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, T2N 4N1 Canada.

[3] To whom correspondence and reprint requests should be addressed at VIDO, 120 Veterinary Road., University of Saskatchewan, Saskatoon, S7N 5E3 Canada. Fax: (306) 966-7478. E-mail: Tikoo@sask.usask.ca.

0042-6822/01 $35.00

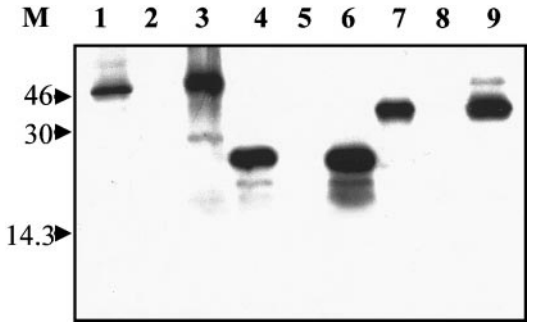

FIG. 1. Immunoprecipitation of proteins synthesized by *in vitro* transcription and translation of plasmids. [^{35}S]Methionine-labeled, *in vitro* transcribed and translated pSP64-PE1A (lanes 7, 9), pSP64-PE1Bs (lanes 4, 6), pSP64-PE1B1 (lanes 1, 3), and pSP64polyA (lanes 2, 5, 8) products before (lanes 3, 6, 9) and after immunoprecipitation with anti-E1A (lanes 7, 8), anti-E1B^{small} (lanes 4, 5), and anti-E1B^{large} (lanes 1, 2) were separated on 10% SDS–PAGE gels under reducing conditions. The positions of the molecular weight markers are shown to the left of the panel.

virus vector and long-term expression of the transgene. Moreover, E1 deleted adenovirus vector provides a significant safety profile to the vaccine as it eliminates the potential for the spread of the vector to nonvaccinated contacts or to the general environment. Earlier, we constructed a replication-defective PAV-3 [E1A (803 bp) and E3 (597 bp) deletion; Reddy *et al.,* 1999b]. Interestingly, this virus replicated 2 logs less efficiently than the wild-type PAV-3 in E1 complementing VIDO R1 cells (fetal porcine retina cells transformed with HAV-5 E1; Reddy *et al.,* 1999b). Here, we report the characterization of proteins encoded by the E1 region, construction of a modified E1A deleted PAV-3, which replicates as efficiently as wild-type in E1 complementing VIDO R1 cells, and construction of an E1B^{small} deleted PAV-3, which replicates as efficiently as wild-type in E1 complementing VIDO R1 or noncomplementing swine testicular (ST) cells. In addition, we describe the construction of E1A + E1B^{small} + E3 deleted PAV-3 and use of this vector to express green fluorescent protein (GFP).

RESULTS

Characterization of PAV-3 E1 proteins

In order to identify and characterize the proteins encoded by the E1 region of PAV-3, we made anti-E1A, anti-E1B^{small}, and anti-E1B^{large} sera by immunizing rabbits with 300 μg of gel-purified glutathione S-transferase (GST)-protein fusions. Sera collected after the final boost were analyzed by *in vitro* transcription and translation assays to determine specificity of the antibodies in the rabbit sera. The plasmids pSP64-PE1A, pSP64-PE1Bs, and pSP64-PE1B1 were generated in which the coding sequence of E1A, E1B^{small}, and E1B^{large}, respectively, was placed downstream of the SP6 promoter. *In vitro* translation of pSP64-PE1A RNA resulted in the synthesis of a polypeptide of 35 kDa (Fig. 1, lane 9) which was recognized by anti-E1A serum (Fig. 1, lane 7). *In vitro* translation of pSP64-PE1Bs RNA resulted in the synthesis of a polypeptide of 23 kDa (Fig. 1, lane 6) which was recognized by anti-E1B^{small} serum (Fig. 1, lane 4). Similarly, *in vitro* translation of pSP64-E1B1 RNA resulted in the synthesis of a polypeptide of 53 kDa (Fig. 1, lane 3) which was recognized by anti-E1B^{large} serum (Fig. 1, lane 1). These proteins were not immunoprecipitated with anti-E1A serum (Fig. 1, lane 8), anti-E1B^{small} serum (Fig. 1, lane 5), or anti-E1B^{large} serum (Fig. 1, lane 2) from reactions in which pSP64polyA (negative control plasmid) was translated *in vitro.*

To further characterize the proteins and to confirm the specificity of the antisera, radioimmunoprecipitation assays (RIPAs) were performed. Anti-E1A serum detected a protein of 35 kDa in PAV-3 infected cells (Fig. 2A, lanes 1 and 2) but not in mock-infected cells (Fig. 2A, lane 3). The 35-kDa protein was detected at 6 h (Fig. 2A, lane 1) and 24 h (Fig. 2A, lane 2) postinfection. Anti-E1B^{small} detected a protein of 23 kDa in PAV-3 infected cells (Fig. 2B, lanes 1 and 2) but not in mock-infected cells (Fig. 2B, lane 3). The 23-kDa protein was detected at 6 h (Fig. 2B, lane 1) and 24 h (Fig. 2B, lane 2) postinfection. Similarly, anti-E1B^{large} serum detected a protein of 53 kDa in PAV-3 infected cells (Fig. 2C, lanes 1 and 2) but not in mock-infected cells. The 53-kDa protein was detected at 6 h (Fig. 2C, lane 1) and 24 h (Fig. 2C, lane 2) postinfection.

Generation of PAV-3 E1 deletion/insertion mutants

Taking advantage of homologous recombination in *E. coli* strain BJ5183, initially we constructed three full-length plasmids: (a) pFPAV211, containing deletions in the E1A (nt 530–1230) and E3 (nt 28,112–28,709) regions (Fig. 3A); (b) pFPAV212, containing deletions in the E1B^{small} (nt 1460–1820) and E3 (nt 28,112–28,709) regions (Fig. 3B); and (c) pFPAV507, containing a TPS (triple phase stop) codon in E1B^{large} (nt 2190) and deletion of the E3 (nt 28,112–28,709) region (Fig. 3C). The *Pac*I digested pFPAV211 or pFPAV212 plasmid DNAs were transfected

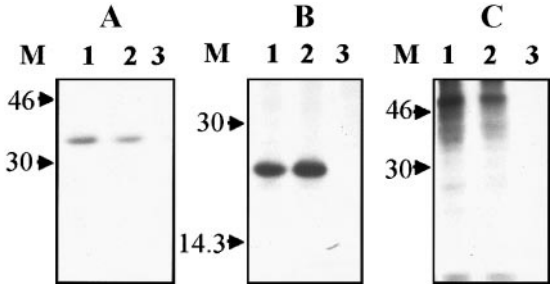

FIG. 2. *In vivo* immunoprecipitation of E1 proteins. Proteins from the lysates of [^{35}S]methionine–cysteine-labeled mock-infected (lane 3) or PAV3 infected (lane 1, 6 h postinfection; lane 2, 24 h postinfection) VIDO R1 cells were immunoprecipitated with anti-E1A serum (A), anti-E1B^{small} serum (B), or anti-E1B^{large} serum (C) and separated on 10% SDS–PAGE under reducing conditions. The positions of the molecular weight markers are indicated to the left of each panel.

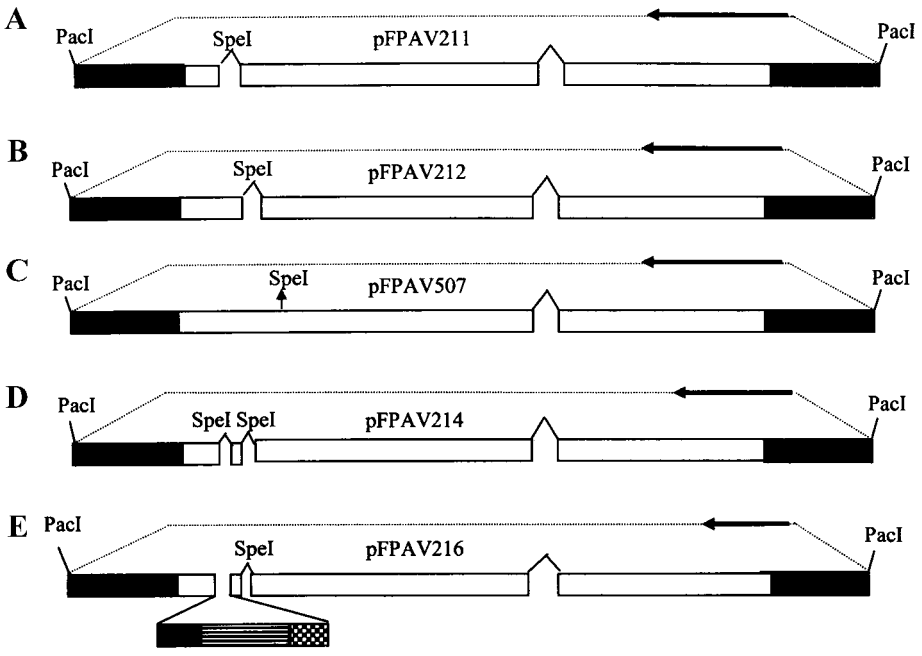

FIG. 3. Schematic representation of full-length genomic DNA in plasmids. (A) plasmid pFPAV211; (B) plasmid pFPAV212; (C) plasmid pFPAV507; (D) plasmid pFPAV214; (E) plasmid pFPAV216. The origin of DNA sequences is as follows: BAV-3 genome (open box); ITR (filled box); AmpR gene (arrow); plasmid DNA (broken line). HCMV promoter (■); GFP gene (▤); BGH poly(A) (▩). The plasmid maps are not drawn to scale.

into VIDO R1 cells and produced cytopathic effects in 10–14 days. However, repeated transfection of VIDO R1 cells with *Pac*I digested pFPAV507 DNA did not produce any cytopathic effects. The transfected cell monolayers were collected and freeze–thawed, and recombinant viruses were plaque-purified and propagated in VIDO R1 cells. The recombinant PAVs were named PAV211 (E1A + E3 deletion) and PAV212 (E1B^{small} + E3 deletion). The viral DNA was isolated from virus infected cells by the Hirt extraction method (Hirt, 1967) and analyzed by agarose gel electrophoresis after digestion with restriction enzymes. Since PAV211 and PAV212 genomes contain an additional *Spe*I site in place of the E1A or E1B^{small} regions, respectively, the recombinant viral DNAs were digested with *Spe*I. As seen in Fig. 4A, compared with wild-type PAV-3 (lane 3), the PAV211 (lane 1) and PAV212 (lane 2) genomes contain an additional expected band of 530 and 1460 bp, respectively.

The ability of PAV211 and PAV212 to produce E1A and E1B^{small} or DNA binding protein (DBP) was tested by Western blot analysis of these proteins from lysates of virus infected ST cells using PAV-3 E1A-, E1B^{small}-, or DBP-specific antisera (Zhou *et al.,* in press,a). Wild-type PAV-3 (Fig. 5C, lane 3) or PAV212 (Fig. 5C, lane 1) infected cells produced an E1A protein of 35 kDa. No such protein was detected in PAV211 (Fig. 5C, lane 2) infected cells. Similarly, wild-type PAV-3 (Fig. 5B, lane 3) and PAV212 (Fig. 5B, lane 1) produced a DBP of 50 kDa. No such protein was detected in PAV211 (Fig. 5B, lane 2) infected cells. In addition, wild-type PAV-3 (Fig. 5A, lane 3) infected cells produced an E1B^{small} protein of 23 kDa (Fig. 5B, lane 3). However, no such protein was detected in PAV211 (Fig. 5A, lane 2) or PAV212 (Fig. 5A, lane 1) infected cells.

Construction of E1A + E1B^{small} + E3 deletion mutant of PAV-3

In order to increase the insertion capacity of the PAV-3 vector, a full-length plasmid pFPAV214 (Fig. 3D) carrying deletions in E1A (nt 530–1230), E1B^{small} (nt 1460–1820), and E3 (nt 28,112–28,709) was constructed by homologous recombination in *E. coli* BJ5183. Transfection of VIDO R1 cells with *Pac*I digested plasmid pFPAV214 DNA produced cytopathic effects in 7–10 days. The recombinant PAV-3, named PAV214, was plaque-purified and expanded in VIDO RI cells. The viral DNA was extracted and analyzed by agarose gel electrophoresis after digestion with *Nhe*I. As seen in Fig. 4B, the wild-type PAV-3 had a fragment of 1.430 kb (lane 2) that was missing in PAV214, which instead had a fragment of 0.737 kb (lane 1).

Construction of E1A + E1B^{small} + E3 deleted PAV-3 expressing GFP

In order to determine whether the PAV214 genome (E1A, E1B^{small}, and E3 deleted) is useful for expression of foreign genes, we constructed recombinant PAV-3 expressing GFP. The full-length GFP gene [flanked by the human cytomegalovirus (HCMV) promoter and bovine growth hormone (BGH) poly(A) signal] was inserted into the E1A region of pFPAV214 in the same transcriptional orientation as E1 (using the homologous recombination machinery of *E. coli*), creating plasmid pFPAV216 (Fig. 3E). The *Pac*I digested pFPAV216 DNA was transfected

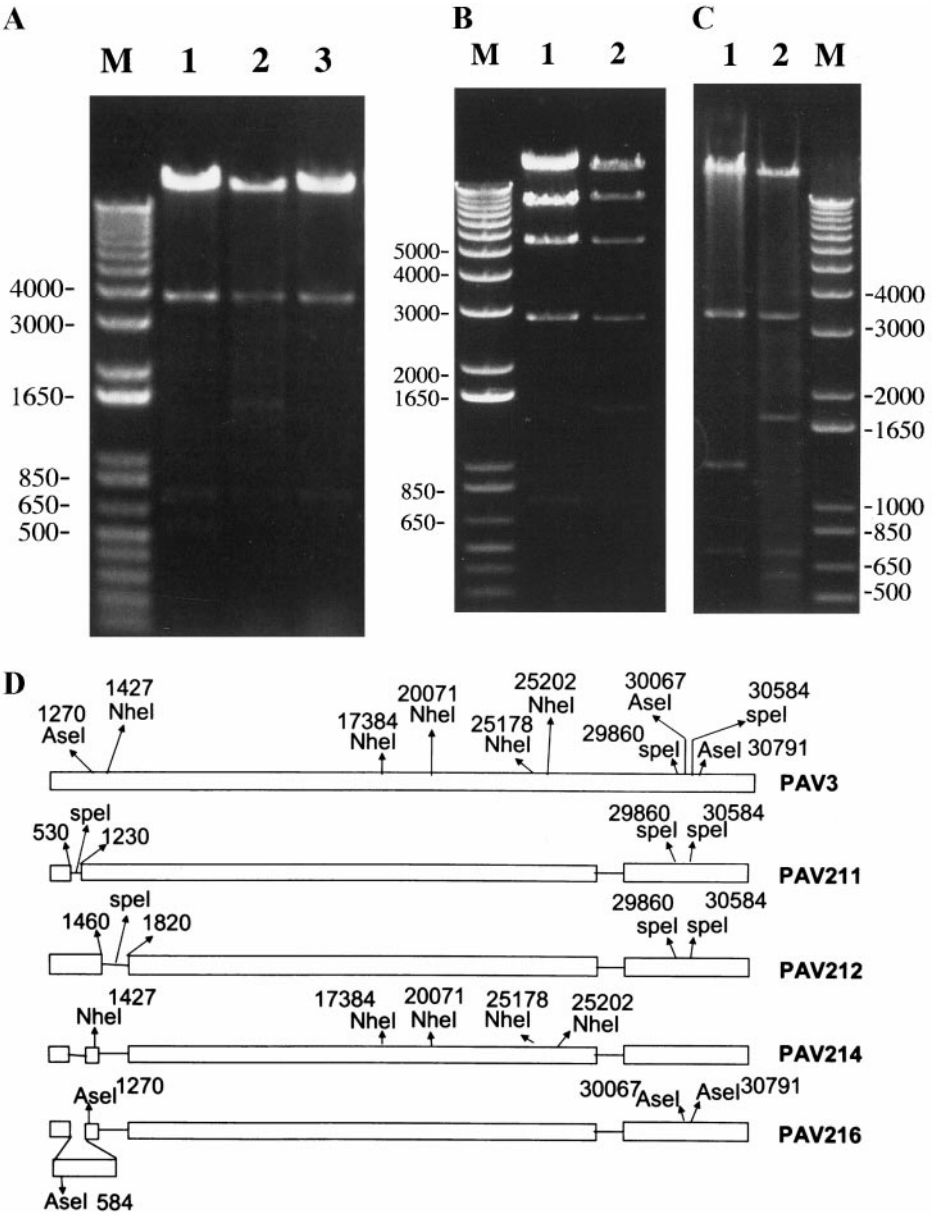

FIG. 4. Restriction enzyme analysis of recombinant PAV-3 genome. (A) The viral DNAs were extracted from VIDO R1 cells infected with PAV211 (lane 1), PAV212 (lane 2), or wild-type PAV-3 (lane 3) and digested with *Spe*I. Sizes of markers (M) are shown in basepairs. (B) The viral DNAs were extracted from VIDO R1 cells infected with PAV214 (lane 1) or wild-type PAV-3 (lane 2) and digested with *Nhe*I. Sizes of markers (M) are shown in basepairs. (C) The viral DNAs were extracted from VIDO R1 cells infected with PAV216 (lane 2) or wild-type PAV-3 (lane 1) and digested with *Ase*I. Sizes of markers (M) are shown in basepairs. (D) Schematic representation of full-length genomes of PAV-3s depicting selected restriction enzyme sites.

into VIDO R1 cells to isolate recombinant virus PAV216. The viral DNA was extracted and analyzed by agarose gel electrophoresis after digestion with restriction enzyme. Since there is an *Ase*I site in the CMV promoter, insertion of the GFP transcription cassette in the E1A region of the PAV214 genome was confirmed by *Ase*I digestion. As seen in Fig. 4C, wild-type PAV-3 had a fragment of 1.274 kb (lane 1) that is missing in PAV216, which instead had two fragments of 0.584 and 1.739 kb (lane 2). Expression of GFP was confirmed by Western blot using GFP-specific polyclonal antibody (Clontech). As seen in Fig. 6, the GFP could be detected in PAV216 infected VIDO R1 cells at 24 (lane 4) and 48 hpi (lane 5). The size of GFP expressed in cells infected with virus is similar to that of purified GFP (lane 2), which is 28 kDa. No such protein could be detected in mock-infected cells (lane 1) or wild-type PAV-3 infected cells (lane 3).

Growth kinetics of PAV211, PAV212, PAV214, and PAV216

In order to determine the importance of E1A and E1B^{small} in viral replication, the ability of mutant viruses to grow in VIDO R1 and ST cells was compared to that of wild-type PAV-3. Virus infected cells were harvested at different times postinfection and freeze–thawed three

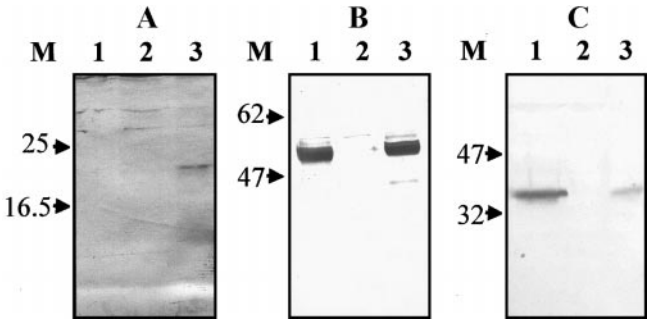

FIG. 5. Western blot analysis of PAV-3 protein expression in mutant infected cells. Proteins from wild-type PAV3 (lane 3), PAV211 (lane 2), or PAV212 (lane 1) infected ST cells were separated by 12.5% SDS–PAGE under reducing conditions and transferred to nitrocellulose. The separated proteins were probed in Western blots by anti-E1A (C), anti-E1B^{small} (A), or anti-DBP (B). The positions of the molecular weight markers are shown to the left of each panel.

times and the cell lysates were analyzed for virus titer by DBP detection assay (Zhou *et al.,* in press).

Wild-type PAV-3 titer was 5.2×10^7 infectious units (IU)/ml at 72 h postinfection (hpi) on VIDO R1 cells. The titers of mutant viruses were between 2×10^7 and 3.2×10^7 IU/ml, values which are quite similar to that of wild-type PAV-3 virus. Therefore, PAV vectors with deletions in E1A and/or E1B^{small} did not have any effect on the ability of PAV-3 to propagate in VIDO R1 cells (E1 complementing cell line) (Fig. 7A). In contrast, we could not observe any progeny virus production in PAV211, PAV214, and PAV216 infected ST cells (E1 noncomplementing). The virus titers at 72 hpi were never more than 2×10^5 IU/ml, which was lower than the amount of input virus (Fig. 7B). All of these three viruses carry deletions in the E1A region. Most notably, mutant virus PAV212 that carried deletions in the E1B^{small} region was able to grow both in complementing and noncomplementing cell lines (Figs. 7A and 7B). At 72 hpi the production of PAV212 in VIDO R1 and ST cells was 3.3×10^7 and 3.9×10^7 IU/ml, respectively.

DISCUSSION

Adenoviruses have been shown to be excellent mammalian cell expression vectors, which are currently considered as one of the most efficient viral vectors for gene delivery *in vivo.* We (Reddy *et al.,* 1999a,b,c; Zakhartchouk *et al.,* 1998, 1999) and others (Hofmann *et al.,* 1999; Khatri *et al.,* 1997; Klonjkowski *et al.,* 1997; Michou *et al.,* 1999; Sheppard *et al.,* 1998; Xu *et al.,* 1997; Xu and Both, 1998) are extending the range of adenoviral vectors for use in vaccination and human gene therapy by developing adenoviruses of animal origins as vectors. In addition, these species-specific adenoviral vectors are ideal candidates for developing novel recombinant vaccines for animals. Previously, we have reported the development of replication-competent (Reddy *et al.,* 1999a) and replication-defective (Reddy *et al.,* 1999b) PAV-3 vectors. In this report, we describe the characterization of E1 proteins. In addition, we describe the development of E1A + E3, E1B^{small} + E3, and E1A + E1B^{small} + E3 deleted PAV-3 vectors and demonstrate that E1A + E1B^{small} + E3 deleted PAV-3 can be used for the expression of foreign genes.

The E1 mRNAs of PAV-3 (Reddy *et al.,* 1998a) have the potential to code for three proteins including 229R (E1A),

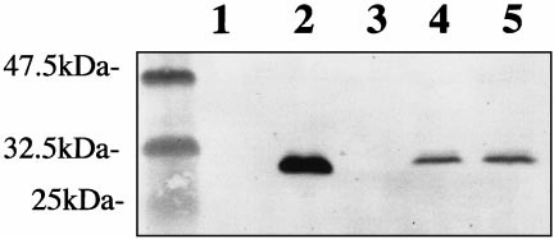

FIG. 6. Western blot analysis of GFP expression. Proteins from purified GFP (lane 2) or mock (lane 1), wild-type PAV-3 (lane 3), and PAV216 (lane 4 and 5) infected VIDO R1 cells harvested at 24 hpi (lane 3, 4) and 48 hpi (lane 5) were separated by 10% SDS–PAGE under reducing conditions and transferred to nitrocellulose. The separated proteins were probed Western blots by anti-GFP polyclonal antibody.

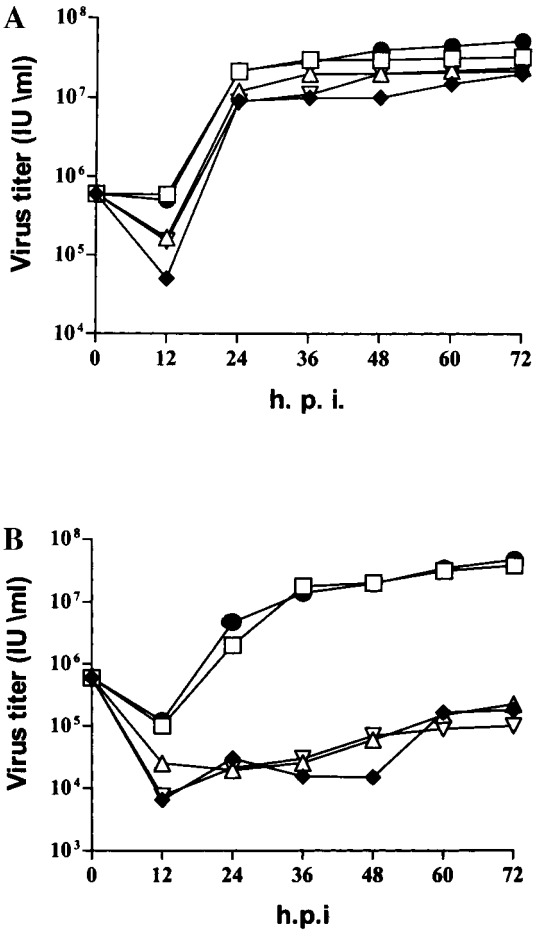

FIG. 7. Virus titers of recombinant and wild-type PAV-3. Near-confluent monolayers of VIDO R1 (A) or ST (B) cells were infected with recombinant or wild-type PAV-3. At different time points postinfection, the cell pellets were freeze–thawed and virus was titrated on VIDO R1 cells as described in the text. PAV211 (◆), PAV212 (□), PAV214 (△), PAV216 (▽), PAV-3 wt. (●).

202R (E1B^{small}), and 474R (E1B^{large}). The 229R protein, synthesized as a 35-kDa protein in PAV-3 infected cells shows homology to the CR2 and CR3 domains of the E1A protein of HAV-2 (Zantema and Van Der Eb, 1995), which are involved in the efficient transcription and activation of other promoter elements in the genome of adenovirus. The 202R protein, produced as a 23-kDa protein in PAV-3 infected cells, shows homology to the central and amino-terminal regions of the E1B^{small} protein of HAV-2 (White *et al.,* 1992), which are important in the anti-apoptotic function of the protein. The 474R protein, synthesized as 53-kDa protein in PAV-3 infected cells, shows homology to domains of the 55K protein of HAV-5 (Rubenwolf *et al.,* 1997), which are involved in protein–protein interactions. However, despite some amino acid homologies among E1 proteins of different adenoviruses, functional dissimilarities have been reported (Abrahamsen *et al.,* 1997; Ying *et al.,* 1998).

Recombinant PAV211 lacking the E1A (nt 530–1230) and part of E3 (nt 28,112–28,709) coding regions grew to titers similar to wild-type PAV-3 in VIDO R1 cells. In contrast, recombinant PAV201 containing deletions in E1A (nt 407–1210) and E3 (nt 28,112–28,709) grew to titers of two logs less than wild-type PAV-3 in E1 expressing VIDO R1 cells (Reddy *et al.,* 1999b). This suggests that deletion of 123 bp (nt 407–530) results in the reduced yield of PAV201 in VIDO R1 cells. It is possible that this region may contain some enhancer sequences, which may affect the early viral gene expression. Alternatively, it is possible that this region may contain some packaging signal(s), which may affect the efficiency of virion DNA encapsidation. Further experiments are needed to support or refute these speculations.

Recombinant PAV211 virus lacking the E1A (nt 530–1230) and part of E3 (nt 28,112–28,709) coding regions failed to produce infectious progeny virus in E1 nonexpressing ST cells. In addition, no expression of early region genes was detected in PAV211 virus infected ST cells. These results confirm our earlier observation (Reddy *et al.,* 1999b) and further suggest that the E1A protein is required for the transactivation of other PAV-3 early genes. Similar results have been reported for the E1A protein of BAV-3 (Zhou *et al.,* 2001), HAV-5 (Berk, 1986), or CAV-2 (Klonjkowski *et al.,* 1997; Kremer *et al.,* 2000). However, E1A of MAV-1 is not required for the transactivation of other early genes (Ying *et al.,* 1998).

It is possible that PAV211 virus detected in ST cells is the progeny virus produced at low levels. However, no expression of early region genes was detected in PAV211 virus infected ST cells, which is thought to be required for the production of progeny virus. Alternatively, it is possible that the E1A gene has been restored through PAV211 virus stock preparation in the VIDO R1 cells by homologous recombination. However, acquisition of the E1A gene by homologous recombination is a very remote possibility as VIDO R1 cells contain the HAV-5 E1 region DNA, which does not show any significant similarity to the nucleotide sequence of the E1 region retained in PAV211.

Recombinant PAV212 containing deletions in E1B^{small} (nt 1460–1820) and part of E3 (nt 28,112–28,709) grew to titers similar to wild-type PAV-3 in E1 expressing VIDO R1 cells. In the absence of E1B^{small}, both early and late region genes were expressed. In addition, PAV212 grew to titers similar to wild-type PAV-3 in E1 nonexpressing ST cells. These results suggest that the E1B^{small} gene product does not impose any significant regulatory effects on viral growth or on the expression of the viral early genes in ST cells. Earlier studies using E1B^{small} mutants of HAV-5 (Hu and Hsu, 1997; White and Stillman, 1987) and BAV-3 (Zhou *et al.,* 2001) have suggested that the requirement of E1B^{small} protein for viral replication depends on the type of cultured cell line used. Although we have not found a significant difference in the replication of PAV212 in the few available porcine cell lines (data not shown), nevertheless we need to test more porcine cell lines.

The construction of PAV214 was the first step toward the development of a PAV-3 vector containing deletions in the E1A, E1B^{small}, and E3 regions. PAV214 contains a 700-bp E1A deletion (nt 530–1230), a 360-bp E1B^{small} deletion (nt 1460–1820), and a 597-bp E3 deletion (nt 28,112–28,709) and therefore should be able to accommodate 3.5-kb foreign DNA, an improvement over the capacity of currently available PAV-3 vector (Reddy *et al.,* 1999b). The construction of PAV216 further demonstrated the feasibility of using this vector system for foreign gene expression. These viruses could be propagated in VIDO R1 cells with growth kinetics similar to those of wild-type, indicating that neither the extended deletion (E1A, E1B^{small}, E3) nor foreign gene insertion alters the viability of the viruses.

It has been suggested that E1B may not be required for complementation of E1A and E1B deleted HAV-5 (Imler *et al.,* 1996). However, repeated attempts to extend the deletion further in the E1 region proved futile as we could not rescue a PAV-3 vector with either gene deletion or insertional inactivation (TPS codon) of the E1B^{large} gene even in VIDO R1 (E1 complementing) cells. Similarly, E1B deleted HAV-7a could not be isolated using 293 (E1 complementing; Abrahamsen *et al.,* 1997) cells. It is possible that the human E1B 55-kDa protein does not complement the porcine E1B^{large} defect of PAV-3. Since the E1B^{large} gene product of HAV-5 interacts with both host and viral proteins, the absence of PAV-3-specific E1B^{large} interactions in VIDO R1 cells (fetal porcine retina cells transformed with HAV-5 E1) may be responsible for not isolating E1B^{large} defective PAV-3.

MATERIALS AND METHODS

Cells and viruses

VIDO R1 (Reddy *et al.,* 1999b) and ST cells were grown and maintained in minimum essential medium (MEM)

supplemented with 10% fetal bovine serum (FBS). The mutant PAV-3 and wild-type PAV-3 (strain 6618) were propagated and titrated in VIDO R1 cells (Reddy *et al.,* 1999b).

GST fusion and antibody production

The plasmid pE1A was created by amplifying part of E1A (nt 556–1222) by PCR and ligating in-frame to the GST gene in plasmid pGEX-5X-3. To create plasmid pE1Bs, part of the $E1B^{small}$ ORF (nt 1470–2070) was amplified by PCR and ligated in-frame to the GST gene in plasmid pGEX-5X-3. The plasmid pE1B1 was created by amplifying the complete $E1B^{large}$ ORF (nt 1831–3250) by PCR and ligated in-frame to the GST gene in plasmid pGEX-5X-3. The junctions of the sequences encoding GST–E1A, GST–$E1B^{small}$, or GST–$E1B^{large}$ were sequenced to ensure that the coding domains are in-frame. The competent *E. coli* strain BL121 was transformed with pE1A, pE1Bs, or pE1B1 plasmids. The fusion protein(s) was induced by addition of 0.1 M isopropyl-β-D-thiogalactoside and purified using sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). Rabbits were immunized subcutaneously with 300 μg of gel-purified GST–E1A, GST–$E1B^{small}$, or GST–$E1B^{large}$ fusion proteins in Freund's complete adjuvant followed by three injections in Freund's incomplete adjuvant at 4-week intervals.

In vitro transcription and translation

The complete coding regions of E1A, $E1B^{small}$, and $E1B^{large}$ were individually cloned into the *Sma*I site of plasmid pSP64 polyA, creating plasmids pSP64-PE1A, pSP64-PE1Bs, and pSP64-PE1B1, respectively. The plasmid DNAs were transcribed and translated *in vitro* by using a rabbit reticulocyte lysate-coupled transcription–translation system in the presence of 50 μCi of [^{35}S]methionine. The *in vitro* translated proteins were analyzed with or without immunoprecipitation with the protein-specific polyclonal rabbit serum.

Construction of PAV-3 recombinant plasmids

The recombinant plasmid vectors were constructed by standard procedures using restriction enzymes and other DNA modifying enzymes.

Construction of plasmid pFPAV211. A 9.225-kb *Xho*I fragment [containing vector backbone plus the left (nt 1 to 4159) and right (nt 31,053 to 34,094) termini of the PAV-3 genome] isolated from plasmid pFPAV200 (Reddy *et al.,* 1999a) was religated, creating plasmid pPAVXhoIRL. Nucleotide numbers of the PAV-3 genome referred to in this report are given according to GenBank Accession No. AF083132. To delete the E1A region, the PAV-3 genome between nt 0 and 531 was amplified by using primers YZ-13, 5′-ATA GGC GTA TCA CGA GGC-3′, and YZ-14, 5′-CTG GAC TAG TCT GTT CCG CTG AGA GAA AAC-3′, and plasmid pPAVXhoIRL DNA as a template in a PCR. The PAV-3 genomic DNA between nt 1231 and 1529 was amplified by using primers YZ-15, 5′-GTG GAC TAG TCT-CAT GCA GCG AACAAC C-3′, and YZ-16, 5′-GTA CTA TCA CCT TCC TAA GG-3′, and plasmid pPAVXhoIRL DNA as a template in a PCR. The product of the first PCR was digested with *Bam*HI–*Spe*I and gel-purified. The second PCR product was digested with *Spe*I–*Bsu*36 and gel-purified. The two gel-purified fragments were cloned into *Bam*HI and *Bsu*36 digested plasmid pPAVXhoIRL in a three-way ligation. The resulting plasmid, pYZ20, carried a 700-bp (nt 530 to 1230) deletion in the E1A region and an engineered *Spe*I site. The recombinant PAV-3 genome containing deletions in the E1A and E3 regions (pFPAV211) was generated by homologous DNA recombination in *E. coli* BJ 5183 between *Xho*I linearized pYZ20 and genomic DNA of PAV-3 E3 (Reddy *et al.,* 1999a; Fig. 3A).

Construction of plasmid pFPAV212. A 633-bp fragment (nt 827 to 1460) isolated by PCR amplification (using oligonucleotides YZ-17, 5′-ACA GTA ATG AGG AGG ATA TC-3′, and YZ-18, 5′-TAG GAC TAG TCC CAC AGA AAA AGA AAA GG-3′, as primers and plasmid pPAVXhoIRL as a template) was digested with *Eco*RV–*Spe*I and gel-purified. A 403-bp fragment (nt 1820 to 2223 of the PAV-3 genome) isolated by PCR amplification (using oligonucleotides YZ-19, 5′-ATG GAC TAG TCT TCT GGT GCC GCC ACT A-3′, and YZ-20, 5′-CCT AAT CTG CTC AAA GCT G-3′, as primers and plasmid pPAVXhoIRL DNA as a template) was digested with *Spe*I–*Eco*47III and gel-purified. A 6.947-kb *Xho*I–*Stu*I fragment of plasmid pPAVXhoIRL was blunt end repaired with T4 polymerase and religated to create plasmid pYZ9a. The two gel-purified DNA fragments were ligated to *Eco*RV–*Eco*47III digested plasmid pYZ9a in a three-way ligation. The resulting plasmid pYZ21 contains a 360-bp deletion (nt 1460–1820) in the $E1B^{small}$ region and an engineered *Spe*I site. Finally, a 5.506-kb *Hpa*I–*Asp*I fragment of pYZ21 was ligated to the 3.374-kb *Hpa*I–*Asp*I fragment of pPAVXhoIRL to create plasmid pYZ21a. The recombinant PAV-3 genome containing deletions in the $E1B^{small}$ and the E3 regions (pFPAV212) was generated by homologous DNA recombination in *E. coli* BJ5183 between *Xho*I linearized pYZ21a and the genomic DNA from PAV E3 (Reddy *et al.,* 1999a; Fig. 3B).

Construction of plasmid pFPAV507. Plasmid pPAVXhoIRL was digested partially with *Eco*47III and ligated to the *Spe*I linker (TPS codon). Plasmid pYZ9 containing the *Spe*I linker inserted in the $E1B^{large}$ ORF was selected. The recombinant PAV-3 genome containing a deletion in E3 and an insertion in $E1B^{large}$ (pFPAV507) was generated by homologous DNA recombination machinery in *E. coli* BJ5183 between *Xho*I linearized pYZ9 and the genomic DNA from PAV E3 (Reddy *et al.,* 1999a; Fig. 3C).

Construction of plasmid pFPAV214. A 0.591-kb *Bam*HI–*Ase*I fragment was excised from plasmid pYZ20 and ligated to 5.309-bp *Bam*HI–*Ase*I (partial) digested pYZ21 to create plasmid pYZ36. Finally, a 4.813-kb *Hpa*I–*Asp*I fragment excised from plasmid pYZ36 was ligated to the

3.373-kb *Hpa*I–*Asp*I fragment of plasmid pPAVXhoIRL to create plasmid pYZ37. The recombinant PAV-3 genome containing deletions in the E1A, E1B^{small}, and E3 region (pFPAV214) was generated by homologous recombination in *E. coli* BJ5183 between *Xho*I linearized plasmid pYZ37 and genomic DNA from PAV E3 (Reddy *et al.,* 1999a; Fig. 3D). The full-length plasmid pFPAV214 contained a 727-bp (nt 530–1230) deletion in E1A, a 360-bp (nt 1460–1820) deletion in E1B^{small}, and a 597-bp (nt 27,405–28,112) deletion in E3.

Construction of plasmid pFPAV216. Plasmid pYZ20 was digested with *Spe*I, blunt end repaired with T4 polymerase, and ligated to *Pme*I linker (GTTTAAAC) creating plasmid pYZ39. A 1.424-kb *Ase*I fragment of plasmid pYZ39 was isolated and ligated to the 6.774-kb *Ase*I fragment of pYZ37 to create plasmid pYZ40. Finally, a 1.730-kb *Nru*I–*Pvu*II fragment [containing the HCMV immediate-early promoter, GFP gene, and BGH poly(A) signal] was excised from plasmid pYZ41a (Zhou *et al.,* manuscript in preparation) and ligated to *Pme*I digested pYZ40 to create plasmid pYZ42. The recombinant PAV-3 genome containing the GFP expression cassette insertion in the E1A region of the E1A, E1B^{small}, and E3 deleted regions was generated by homologous recombination in *E. coli* BJ5183 between *Xho*I linearized pYZ42 and genomic DNA from PAV E3 (Reddy *et al.,* 1999a; Fig. 3E).

Transfection and isolation of PAV-3 mutant viruses

VIDO R1 cell monolayers seeded in a 6-well plate were transfected with 5–10 μg of *Pac*I digested pFPAV211, pFPAV212, pFPAV214, pFPAV216, or pFPAV507 recombinant plasmid DNAs using the Lipofectin method (Gibco BRL). After 7–10 days of incubation at 37°C, the transfected cells showing 50% cytopathic effect were collected and freeze–thawed three times. Finally, the recombinant virus was plaque-purified and expanded in VIDO R1 cells.

Virus growth curve

VIDO R1 or ST cells were infected with mutant or wild-type PAV-3 at an m.o.i. of 5. The infected cells, harvested at the indicated times postinfection, were lysed in the infection medium by three rounds of freezing–thawing. Virus titers were determined by serial dilution infections of VIDO R1 cells followed by immunohistochemical detection of DNA binding protein (Zhou *et al.,* in press). Titers were expressed as infectious units in which 1 IU was defined as one positive stained focus at 3 days postinfection.

Western blot

For Western blot, about 1×10^6 VIDO R1 or ST cells were infected with recombinant or wild-type PAV-3 at an m.o.i. of 5. At the indicated times postinfection, the cells were collected and lysed in 100 μl of RIPA buffer (0.15 M NaCl, 50 mM Tris–HCl, pH 8.0, 1% NP-40, 1% deoxycholate, 0.1% SDS). Proteins were resolved on SDS–PAGE under reducing conditions and electrotransferred to a nitrocellulose membrane (Bio-Rad). Nonspecific binding sites were blocked with 1% bovine serum albumin fraction V, and the membrane was probed with the protein-specific rabbit polyclonal serum. The membrane was washed and exposed to goat anti-rabbit IgG conjugated to alkaline phosphatase and developed using an alkaline phosphatase color development kit (Bio-Rad).

Radioimmunoprecipitation

VIDO R1 cells in 6-well plates were infected with wild-type PAV-3 at an m.o.i. of 5. After virus adsorption for 1 h, the cells were incubated in MEM containing 5% FBS. At different times postinfection, the cells were incubated in methionine–cysteine-free MEM for 1 h before being labeled with [^{35}S]methionine–cysteine (100 μCi/well). After 6 or 24 h of labeling, the cells were harvested. Proteins were immunoprecipitated from cells lysed with modified RIPA buffer and analyzed by SDS–PAGE as described previously (Tikoo *et al.,* 1993).

ACKNOWLEDGMENTS

The authors thank Caron Pyne for her technical assistance and the staff of Animal Care Unit at VIDO for their help with animal experiments. This work was supported by grants from Alberta Agriculture Research Institute, Saskatchewan Agriculture Development Fund, and Natural Science and Engineering Research Council of Canada to S.K.T.

REFERENCES

Abrahamsen, K., Kong, H.-L., Masterangeli, A., Brough, D., Lizonova, A., Crystal, R. G., and Falck-Pedersen, E. (1997). Construction of an adenovirus type 7a E1A$^-$ vector. *J. Virol.* **71,** 8946–8951.

Ballay, A., Levrero, M., Buendia, M. A., Tiollais, P., and Perricaudet, M. (1985). In vitro and in vivo synthesis of the hepatitis B virus surface antigen and of the receptor for polymerized human serum albumin from recombinant human adenoviruses. *EMBO J.* **30,** 3861–3865.

Berk, A. J. (1986). Adenovirus promoters and E1A transactivation. *Annu. Rev. Genet.* **20,** 45–79.

Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A., and Mehtali, M. (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J. Virol.* **70,** 4805–4810.

Darbyshire, J. H. (1966). Oncogenicity of bovine adenovirus type 3 in hamsters. *Nature* **211,** 102.

Derbyshire, J. B., Clarke, M. C., and Collins, A. P. (1975). Serological and pathogenicity studies with some unclassified porcine adenoviruses. *J. Comp. Pathol.* **85,** 437–443.

Graham, F. L. (1984). Transformation by and oncogenicity of human adenoviruses. *In* "The Adenoviruses" (H. S. Ginsberg, Ed.), Plenum, New York. 339–398.

Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36,** 59–74.

Hehir, K. M., Armentano, D., Cardoz, L. M., Choquette, T. L., Berthlette, P. B., White, G. A., Couture, L. A., Everton, M. B., Keegan, J., Martin, J. M., Pratt, D. A., Smith, M. P., Smith, A. E., and Wadsworth, S. C. (1996). Molecular characterization of replication-competent variants of adenovirus vectors and genome modifications to prevent their occurrence. *J. Virol.* **70,** 8459–8467.

Hirahara, T., Yauhara, H., Matsui, O., Yamanaka, M., Tanaka, M., and

Fukuyama, S. (1990). Isolation of porcine adenovirus from the respiratory tract of pigs in Japan. *Jpn. J. Vet. Sci.* **52,** 407–409.

Hirt, B. (1967). Selective extraction of polyoma virus DNA from infected mouse cell cultures. *J. Mol. Biol.* **26,** 365–369.

Hitt, M. M., and Graham, F. L. (2000). Adenovirus vectors for human gene therapy. *Adv. Virus Res.* **55,** 479–505.

Hofmann, C., Loser, P., Cichon, Arnold, G., W., Both, G. W., and Strauss, M. (1999). Ovine adenovirus vectors overcome preexisting humoral immunity against human adenovirus in vivo. *J. Virol.* **73,** 6930–6936.

Houweling, A., van den Elsen, P. J., and van der Eb, A. J. (1980). Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. *Virology* **105,** 537–550.

Hu, M., and Hsu, M. (1997). Adenovirus E1B 19K protein is required for efficient DNA replication in U937 cells. *Virology* **227,** 295–304.

Imler, J. L. (1995). Adenovirus vectors as recombinant viral vaccines. *Vaccine* **13,** 1143–1151.

Imler, J. L., Chartier, C., Dreyer, D., Dieterle, A., Sainte-Marie, M., Faure, T., Pavirani, A., and Mehtali, M. (1996). Novel complementation cell lines derived from human lung carcinoma A549 cells support the growth of E1-deleted adenovirus. *Gene Ther.* **3,** 75–84.

Jones, N., and Shenk, T. (1979). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* **17,** 683–689.

Khatri, A., Xu, Z. Z., and Both, G. W. (1997). Gene expression by atypical recombinant ovine adenovirus vectors during abortive infection of human and animal cells *in vitro*. *Virology* **239,** 226–237.

Klonjkowski, B., Gilardi-Hebenstreit, P., Hadchouel, J., Randrianarison, V., Boutin, S., Yeh, P., Perricaudet, M., and Kremer, E. J. (1997). A recombinant E1-deleted canine adenoviral vector capable of transduction and expression of a transgene in human-derived cells and *in vivo*. *Hum. Gene Ther.* **8,** 2103–2115.

Kremer, E. J., Boutin, S., Chillon, M., and Danos, O. (2000). Canine adenovirus vectors: An alternative for adenovirus-mediated gene transfer. *J. Virol.* **74,** 505–512.

Mastrangeli, A., Harvey, B. G., Yao, J., Wolff, G., Kovesdi, I., Crystal, R. G., and Falck-Pedersen, E. E. (1996). "Sero-switch" adenovirus-mediated in vivo gene transfer: Circumvention of anti-adenovirus humoral immune defenses against repeat adenovirus vector administration by changing the adenovirus serotype. *Hum. Gene Ther.* **7,** 79–87.

Michou, A. I., Lehrmann, H., Saltik, M., and Cotton, M. (1999). Mutational analysis of the avian adenovirus CELO, which provides a basis for gene delivery vectors. *J. Virol.* **73,** 1399–1410.

Reddy, P. S., Idamakanti, N., Hyun, B. H., Tikoo, S. K., and Babiuk, L. A. (1999a). Development of porcine adenovirus-3 as an expression vector. *J. Gen. Virol.* **80,** 563–570.

Reddy, P. S., Idamakanti, N., Song, J. Y., Lee, J. B., Hyun, B. H., Park, J. H., Cha, S. H., Tikoo, S. K., and Babiuk, L. A. (1998a). Sequence and transcription map of early region-1 of porcine adenovirus type-3. *Virus Res.* **58,** 97–106.

Reddy, P. S., Idamakanti, N., Song, J. Y., Lee, J. B., Hyun, B. H., Park, J. H., Cha, S. H., Bae, Y. T., Tikoo, S. K., and Babiuk, L. A. (1998b). Nucleotide sequence and transcription map of porcine adenovirus type-3. *Virology* **251,** 414–426.

Reddy, P. S., Idamakanti, N., Babiuk, L. A., Mehtali, M., and Tikoo, S. K. (1999b). Porcine adenovirus-3 as a helper-dependent expression vector. *J. Gen. Virol.* **80,** 2909–2916.

Reddy, P. S., Idamakanti, N., Chen, Y., Whale, T., Babiuk, L. A., Mehtali, M., and Tikoo, S. K. (1999c). Replication-defective bovine adenovirus type 3 as an expression vector. *J. Virol.* **73,** 9137–9144.

Rubenwolf, S., Schutt, H., Nevels, M., Wolf, H., and Dobner, T. (1997). Structural analysis of the adenovirus type 5 E1B 55-kilodalton–E4Orf6 protein complex. *J. Virol.* **71,** 1115–1123.

Sheppard, M., Werner, W., Tsatas, E., McCoy, R., Prowse, S., and Johnson, M. (1998). Fowl adenovirus recombinant expressing VP2 of infectious bursal disease virus induces protective immunity against bursal disease. *Arch. Virol.* **143,** 915–930.

Tikoo, S. K., Parker, M. D., van den Hurk, J. V., Kowalski, J., Zamb, T. J., and Babiuk, L. A. (1993). Role of N-linked glycans in antigenicity, processing and cell surface expression of bovine herpesvirus 1 glycoprotein gIV. *J. Virol.* **67,** 726–733.

White, E., and Stillman, B. (1987). Expression of adenovirus E1B mutant phenotypes is dependent on the host cell and on synthesis of E1A proteins. *J. Virol.* **61,** 426–435.

White, E., Sabbatani, P., Debbas, M., Wold, W. S. M., Kusher, D. I., and Gooding, L. (1992). The 19 kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor α. *Mol. Cell. Biol.* **12,** 2570–2580.

Whyte, P., Ruley, H. E., and Harlow, E. (1988). Two regions of the adenovirus early region 1A proteins are required for transformation. *J. Virol.* **62,** 257–265.

Xu, Z. Z., and Both, G. W. (1998). Altered tropism of an ovine adenovirus carrying the fiber protein cell binding domain of human adenovirus type-5. *Virology* **248,** 156–163.

Xu, Z. Z., Hyatt, A., Boyle, D. B., and Both, G. W. (1997). Construction of ovine adenovirus recombinants by gene insertion or deletion of related terminal region sequences. *Virology* **230,** 62–71.

Ying, B., Smith, K., and Spindler, K. R. (1998). Mouse adenovirus type 1 early region 1A is dispensible for growth in cultured fibroblasts. *J. Virol.* **72,** 6325–6331.

Zakhartchouk, A. N., Pyne, C., Mutwiri, G. K., Papp, Z., Baca-Estrada, M. E., Griebel, P., Babiuk, L. A., and Tikoo, S. K. (1999). Mucosal immunization of calves with recombinant bovine adenovirus type 3: Induction of protective immunity to bovine herpesvirus-1. *J. Gen. Virol.* **80,** 1263–1269.

Zakhartchouk, A. N., Reddy, P. S., Baxi, M., Baca-Estrada, M. E., Mehtali, M., Babiuk, L. A., and Tikoo, S. K. (1998). Construction and characterization of E3-deleted bovine adenovirus type 3 expressing full-length and truncated form of bovine herpesvirus type 1 glycoprotein gD. *Virology* **250,** 220–229.

Zantema, A., and Van Der Eb, A. J. (1995). Modulation of gene expression by adenovirus transformation. *Curr. Top. Microbiol. Immunol.* **199,** 1–23.

Zhou, Y., Pyne, C., and Tikoo, S. K. Characterization of DNA binding protein of porcine adenovirus type 3. *Intervirology,* in press.

Zhou, Y., Reddy, P. S., Babiuk, L. A., and Tikoo, S. K. (2001). Bovine adenovirus type 3 $E1B^{small}$ is essential for growth in bovine fibroblast cells. *Virology* **288,** 264–274.